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Original Article

Design and Synthesis of N-Phenyl-2-(Phenyl-Amino) Acetamide Derivatives as FVIIA Inhibitors for Effective Anticoagulant Activity

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ABSTRACT

Aim: Factor VIIa is a glycosylated disulfide-linked heterodimer that belongs to the serine protease family involved in the coagulation process. Inhibition of factor VIIa is one of the crucial targets for novel anticoagulant agents. Coagulation factor VIIa inhibition has recently attracted attention as an intriguing antithrombotic therapeutic strategy. With the aid of X-ray crystallography and structure-based design, we were able to discover a novel series of N-phenyl-2-(phenyl-amino) acetamide derivatives that exhibited a remarkable affinity for factor VIIa. **Materials and Methods:** The synthesis of 22 compounds was based on the Schotten-Baumann reaction. The synthesized compounds were confirmed by physicochemical, spectroscopic and elemental analysis. *In vitro,* anticoagulant activity was evaluated using prothrombin determination method. **Results:** Compounds 4, 7, 15, 16 and 19 demonstrated good inhibitory anticoagulant activities *in vitro* and showed good docking score *in silico*. N-phenyl-2-(phenyl-amino) acetamide provides a good template for the synthesis of novel and potent anticoagulant derivatives. **Conclusion:** N-phenyl-2-(phenyl-amino) acetamide derivatives can be serving as potential drug compounds for coagulation disorders. The objective of this study was to employ *in silico* molecular docking and *in vitro* anticoagulant activity to design and synthesize structure-based new factor VIIa inhibitors with enhanced potency.

Keywords: N-phenyl-2-(phenyl amino) acetamide, Factor VIIa, Pyrex, X-ray crystallography, Prothrombin time.

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INTRODUCTION

A serine protease enzyme called factor VII A functions in the extrinsic coagulation cascade. The disorders that because thrombosis served as a basis for the development of novel anticoagulants that are selective protease inhibitors.[1-3] additionally, oral bioavailability is inadequate in existing anticoagulant medications, which have issues with bleeding. According to preclinical studies, FVIIa inhibitors may have a higher therapeutic index and be able to overcome the bleeding issues caused by present medications. The development of novel, orally active FVIIa inhibitors can be employed for long-term thrombosis therapy. Targeting upstream proteolytic complexes like FVIIa would offer a better safety advantage, according to reported literature. This study intended to design and synthesize novel N-phenyl-2-(phenyl-amino) acetamide derivatives selectivity to inhibit FVIIa serine enzymes through the virtual screening approach.

MATERIALS AND METHODS

Synthesis

According to Scheme 1, compounds 1-22 were synthesized using the Schotten-Baumann reaction.[4] The reactions comprised three steps. In step I of this process, chloroacetic acid and primary aromatic amine were combined with 10% sodium hydroxide base to yield a product. The resulting product was then refluxed with thionyl chloride for a further 30 min to obtain an acid chloride (Step II) derivative, which was then reacted with a primary aromatic amine to yield derivatives of N-phenyl-2-(phenyl-amino) acetamide (Third Step).

An open capillary method was used to determine the melting points of synthetic compounds. To confirm the presence of amide, amine, carboxylic and acid chloride groups, qualitative analysis tests were carried out. Silica gel-G was used for analytical TLC. The spot was located using UV light (254 nm). Identification and purity of synthesized chemicals were accomplished using RF values. The Jasco-FTIR 4100 device was used to record the

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infrared spectra. NMR spectra were obtained using $CDCl_3$ solvent and a 400 MHz Varian NMR scanner. TMS served as the internal reference.

Step I Procedure

In a conical flask, 5 mL of water, 30 mL of 10% aqueous sodium hydroxide, 0.94 g of chloroacetic acid (0.01 mol) and 1 mol of primary amine were added. Recrystallization of the base product using methanol was accomplished after fifteen min of vigorous shaking (Step I).

Step II Procedure

In a dry conical flask, Step I product and redistilled thionyl chloride were added. The Flask was heated on boiling water bath with occasional shaking for 60 min. The crude product was recovered and recrystallized using a methanol solvent after cooling. (Step II).

Step III Procedure

Primary amine (0.01 mol) was taken in conical flask, 10% sodium hydroxide and step II product were added into it. The conical flask was shaken for 15 min vigorously and further refluxed for 60 min. The product separated as solid and crude product was recrystallized by methanol (Step III).

Synthesis of 2-[(4-acetylphenyl) amino]-N-(4-nitrophenyl) acetamide *(4)*

Yield 91.54%, MP142-144°C, RF 0.89 FTIR: 1315.21, 1523.49 (C-NO_2), 1673.91 (C=O amide), 3085.55, 3166.54, 3463.53 (N-H amide), ^{1}H NMR: δ 2.21, s (3H), 3.63, s (2H), 6.95 (2H, J=8.6, 1.1, 0.5 Hz), 7.35 (2H, J=8.6, 2.3, 0.4 Hz), 7.76 (2H, J=8.6, 1.8, 0.5 Hz), 8.13 (2H, J=8.6, 1.8, 0.4 Hz).

Synthesis of 2-[(3-acetylphenyl) amino]-N-(4-nitrophenyl) acetamide *(7)*

Yield 81.57%, MP180-182°C, RF 0.86 FTIR:1515.78 (C-NO_2), 1604.48 (N-H secondary amine), 1685.48 (C=O amide), 2977.55 ($COCH_3$), 3313.11(N-H amide) ^{1}H NMR: δ 2.26, s (3H), 3.53, s (2H), 7.25-7.46 (4H, 7.31 (dt, J=8.2, 1.3 Hz), 7.35 (J=8.6, 2.3, 0.4 Hz), 7.39 (J=8.4, 8.2, 0.5 Hz)), 7.75-7.95 (2H, 7.81 (J=1.8, 1.3, 0.5 Hz), 7.88 (J=8.4, 1.8, 1.3 Hz)), 8.13 (2H, J=8.6, 1.8, 0.4 Hz).

Synthesis of2-[(4-acetylphenyl) amino]-N-[4-(1, 3-thiazol-2-yl) phenyl] acetamide (15)

Yield 96.00%, MP120-122°C, RF 0.95 FTIR:1481.06, 1523.49(C-NO_2), 1589.06, 16531.48 (N-H secondary amine), 2996.84 ($COCH_3$), 3077.83, 3174.26, 3478.95 (N-H amide) ^{1}H NMR: δ 2.21, s (3H), 3.63, s (2H), 6.89-7.03 (3H, 6.95 (J=8.6, 1.1, 0.5 Hz), 6.97 (J=6.6 Hz)), 7.58-7.83 (7H, 7.63 (d, J=6.6 Hz), 7.72 (J=8.6, 1.6, 0.4 Hz), 7.73 (J=8.6, 1.5, 0.4 Hz), 7.76 (J=8.6, 1.8, 0.5 Hz)).

Synthesis of 2-[(4-nitrophenyl) amino]-N-[4-(1, 3-thiazol-2-yl) phenyl] acetamide (16)

Yield 66.66%, MP 114-116°C, RF 0.91 FTIR: 1592.91, 1627.63 (N-H), 3108.69, 3363.25, 3482.81 (N-H amide) ^{1}H NMR: δ 3.68. S (2H), 6.91-7.04 (3H, 6.97 (J=6.6 Hz), 6.98 (J=7.9, 1.1, 0.5 Hz)), 7.58-7.80 (5H, 7.63 (J=6.6 Hz), 7.72 (J=8.6, 1.6, 0.4 Hz), 7.73 (J=8.6, 1.5, 0.4 Hz)), 8.07 (2H, J=7.9, 1.8, 0.5 Hz).

Synthesis of N-[4-(5-methyl-1, 3, 4-thiadiazol-2-yl) phenyl]-2-[(4-nitrophenyl) amino] acetamide (19)

Yield 96.62% MP130-132°C, RF 0.95 FTIR: 628.68,701.962 (C-S), 1581.34,1627.63 (N-H), 1685.48(C=O amide), 2934.85 ($COCH_3$) ^{1}H NMR: δ 2.43 (3H, s), 3.68 (2H, s), 6.98 (2H, J=7.9, 1.1, 0.5 Hz), 7.61-7.84 (4H, 7.67 (J=8.6, 1.4, 0.4 Hz), 7.78 (J=8.6, 1.5, 0.4 Hz)), 8.07 (2H, J=7.9, 1.8, 0.5 Hz).

Molecular docking analysis

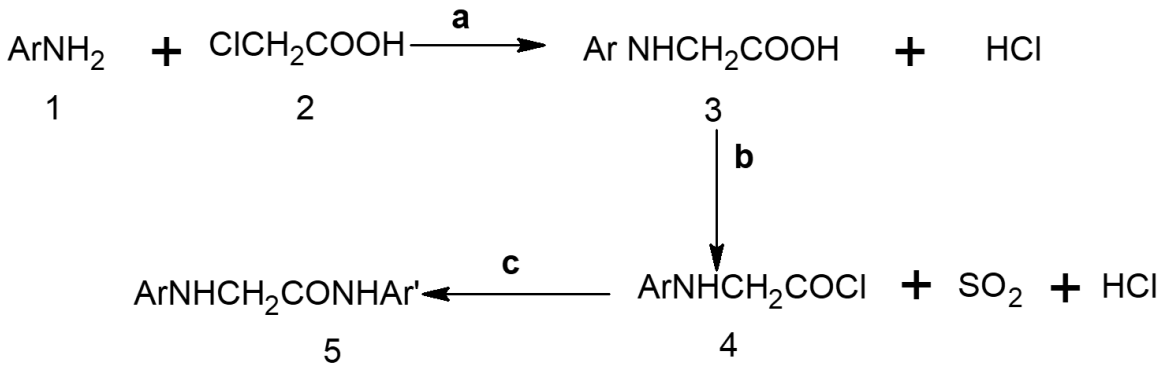
The structure of substituted aromatic acetamide derivatives was drawn in Marvin's sketch.[5] All 2D compounds were cleaned into 3D. The ligand geometries were optimized by using the MMFF94 force field in PyRx. Protein receptor of 1 DAN was downloaded from the RCSB website. The natural substrate and water were removed and hydrogen was added to the receptor using Molegro Virtual Docker.[14,15] The receptor was converted to a macromolecule in PyRx.[6,7]

Anticoagulant activity

The prothrombin determination technique was used to assess the anticoagulant activity of 22 synthesized substances.[8,9] For the determination, 0.1 mL brain thromboplastin was added to 0.1 mL of plasma. After 2 min, pre-warmed calcium chloride solution at 37°C was added to the above solution. The test tube was kept against light to observe the fibrin clot. The stopwatch was paused and the time was noted at the first occurrence (Control). A 0.1 mL solution of the 100 mg/mL test compound has been mixed to 0.1 mL of plasma as a test solution. The solution was incubated for 5 min and elongation in prothrombin time was recorded. The reference drug warfarin was employed.

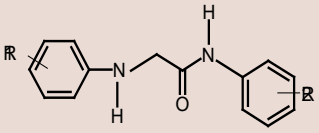
RESULTS

A Conventional method was employed for N-phenyl-2-(phenyl-amino) acetamide derivatives. A total of 22 compounds were prepared (Table 1) and functional groups were confirmed by FTIR. The synthesized substances underwent anticoagulant activity screening. Molecular docking analysis of compounds 4, 7, 10, 15, 16 and 19 had shown strong binding affinity to 1 DAN heavy chain receptor in PyRx-virtual screening software 0.8.[8] Nine conformers in all were generated during the docking process and the conformation with the lowest binding energy and the best scoring pose was selected for further investigation. In Table 2 the binding energies of bioactive compounds were



Scheme 1: Synthetic procedure for 22 compounds of N-phenyl-2-(phenyl-amino) acetamide derivatives. a:10% NaOH, b:$SOCl_2$, c: Ar-NH_2 and 10% NaOH. 1: Primary amine, 2: Chloroacetic acid, 3: Step I Product, 4: Product Step 2, 5: Product Step 3.

Table 1: N-phenyl-2-(phenyl amino) acetamide derivatives.

Structure	Compound	R1	R2	Compound	R1	R2
	1	3-NO_2	4-$COCH_3$	12	3-$COCH_3$	4-$COCH_3$
	2	3 -NO_2	4-NO_2	13	3-NO_2	4-(5-methyl-1,3,4-thiadiazol-2-yl)
	3	3 -NO_2	3 -NO_2	14	4-NO_2	2-methyl-5-[(E)-2-phenylethenyl]-1,3,4-thiadiazole
	4	4-$COCH_3$	4-NO_2	15	4-$COCH^3$	1,3-thiazole
	5	4-$COCH_3$	4-$COCH_3$	16	4-NO_2	1,3-thiazole
	6	4-$COCH_3$	3 -NO_2	17	4-$COCH_3$	4-(5-methyl-1,3,4-thiadiazol-2-yl)
	7	3-$COCH_3$	4-NO_2	18	3-$COCH_3$	4-(5-methyl-1,3,4-thiadiazol-2-yl)
	8	4-NO_2	4-NO_2	19	4-NO_2	4-(5-methyl-1,3,4-thiadiazol-2-yl)
	9	4-NO_2	4-$COCH_3$	20	3-NO_2	2-methyl-5-[(E)-2-phenylethenyl]-1,3,4-thiadiazole
	10	4-NO_2	3 -NO_2	21	3-NO_2	1,3-thiazole
	11	3-$COCH_3$	3 -NO_2	22	1,3-thiazole	1,3-thiazole

demonstrated. Biovia Discovery Studio 3.5[10-13] was used to visualize the non-covalent interactions of protein-compound complexes in both 2D and 3D as represented in Figures 1 and 2.

The anticoagulant activity was screened for all 22 compounds by prothrombin time determination using Quick method. The standard warfarin drug was used for comparison. The normal prothrombin time is 14±2. Compounds 4, 7, 15, 16 and 19 shown 20s, 25s, 16s, 25s and 20s clotting time respectively.

DISCUSSION

The N-H amide group has been determined by the peak at 3482.21 cm^{-1} and the C=O group was affirmed by the peak at 1670.05 cm^{-1}. Characteristics IR spectra of secondary amine at 1631.48 cm^{-1} and CH=CH at 3019.98 cm^{-1} eliciting the presence of strong absorption peaks.

Minimum inhibitory concentration of these compounds was summarized in Table 3.

Compound 4 with a binding affinity of -7.7 kcal/mol, docked with the target receptor through hydrogen bonding with GLU 94 (L), ASN, 95 (L) and with LYS 65 (T), pi sigma interaction with THR 129C (H) and pi alkyl interaction with LYS 46 (T).

Compounds 7 and 15 docked with a binding affinity of -7.7 and -8.4 kcal/mol respectively to a receptor. Compound 7 has hydrogen bonding with PRO 170 I (H), ASN 175 (H), SER 170 (H), HIS 224 (H) and PHE 225 (H), pi cation interaction with LYS 170 D (H) and pi sigma interaction with LEU 169 (H). Compound 15 has shown hydrogen bonding with ASN 95 (L), pi cation bond with LYS 65 (T), Pi alkyl bond with LYS 46(T), pi sigma interaction with THR 129 C (H).

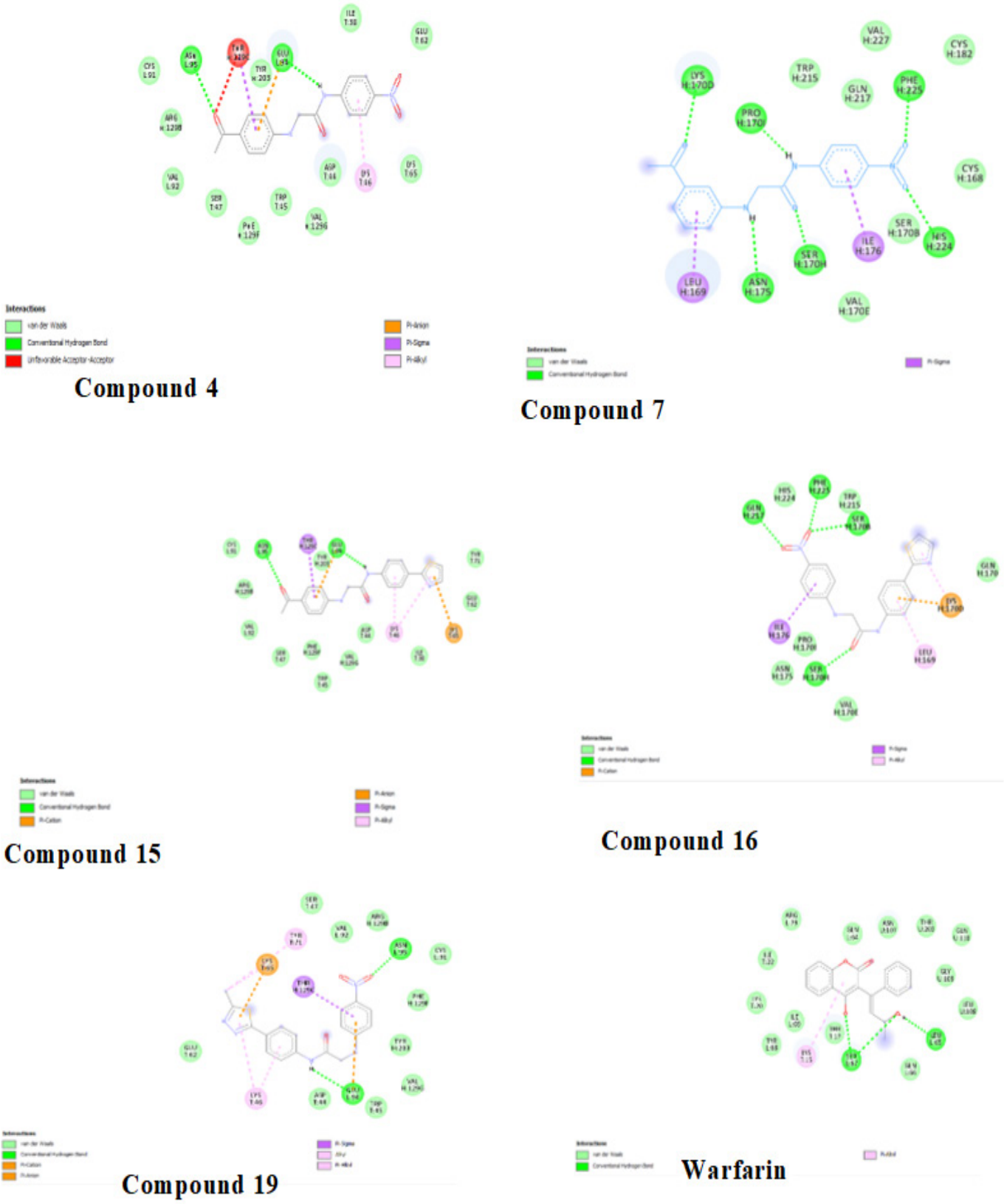
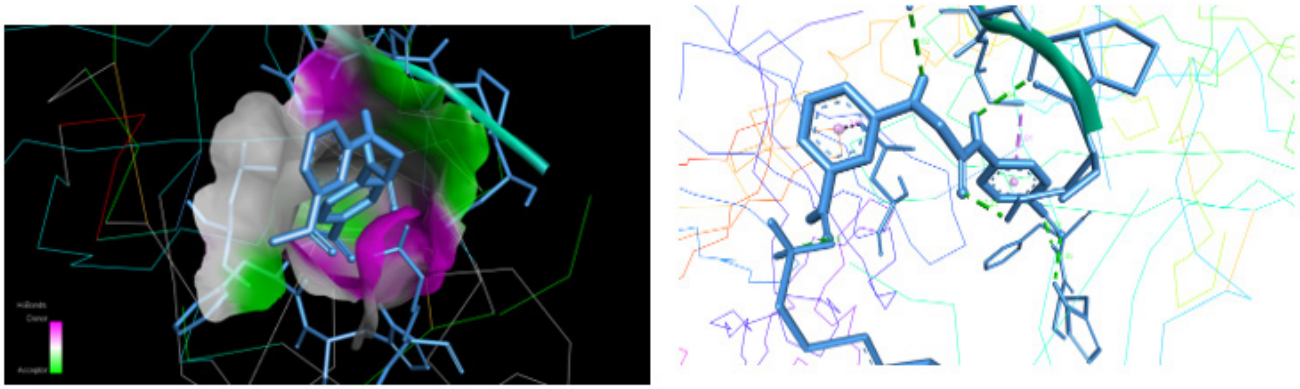
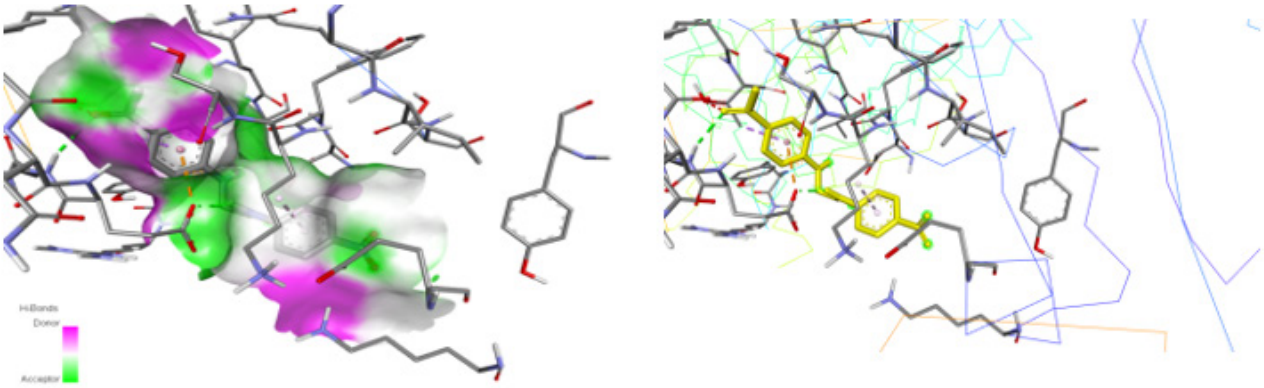


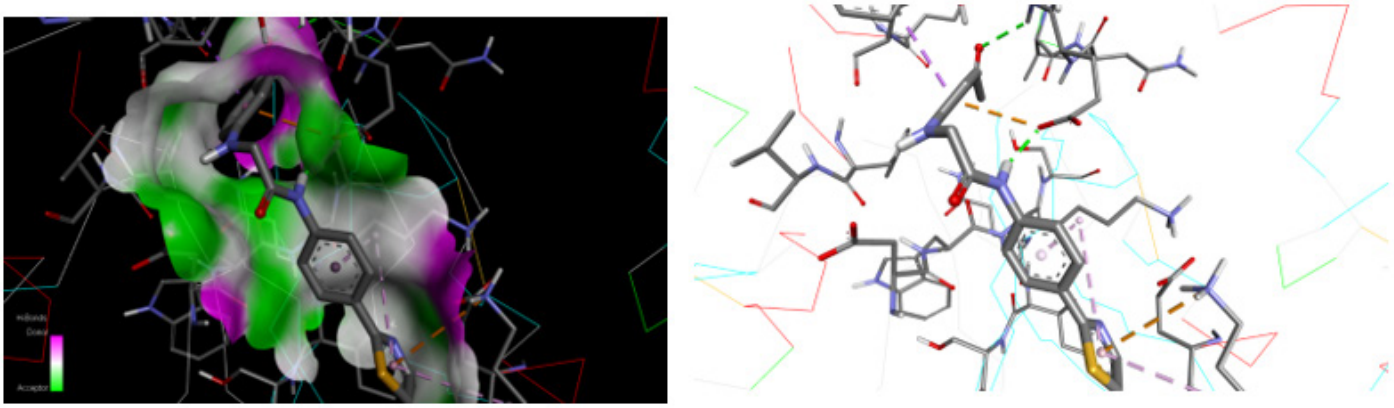
Figure 1: 2D interactions of compounds 4, 7, 15, 16 and 19 with 1DAN Factor VIIa receptor.



Compound 7



Compound 4



Compound 15

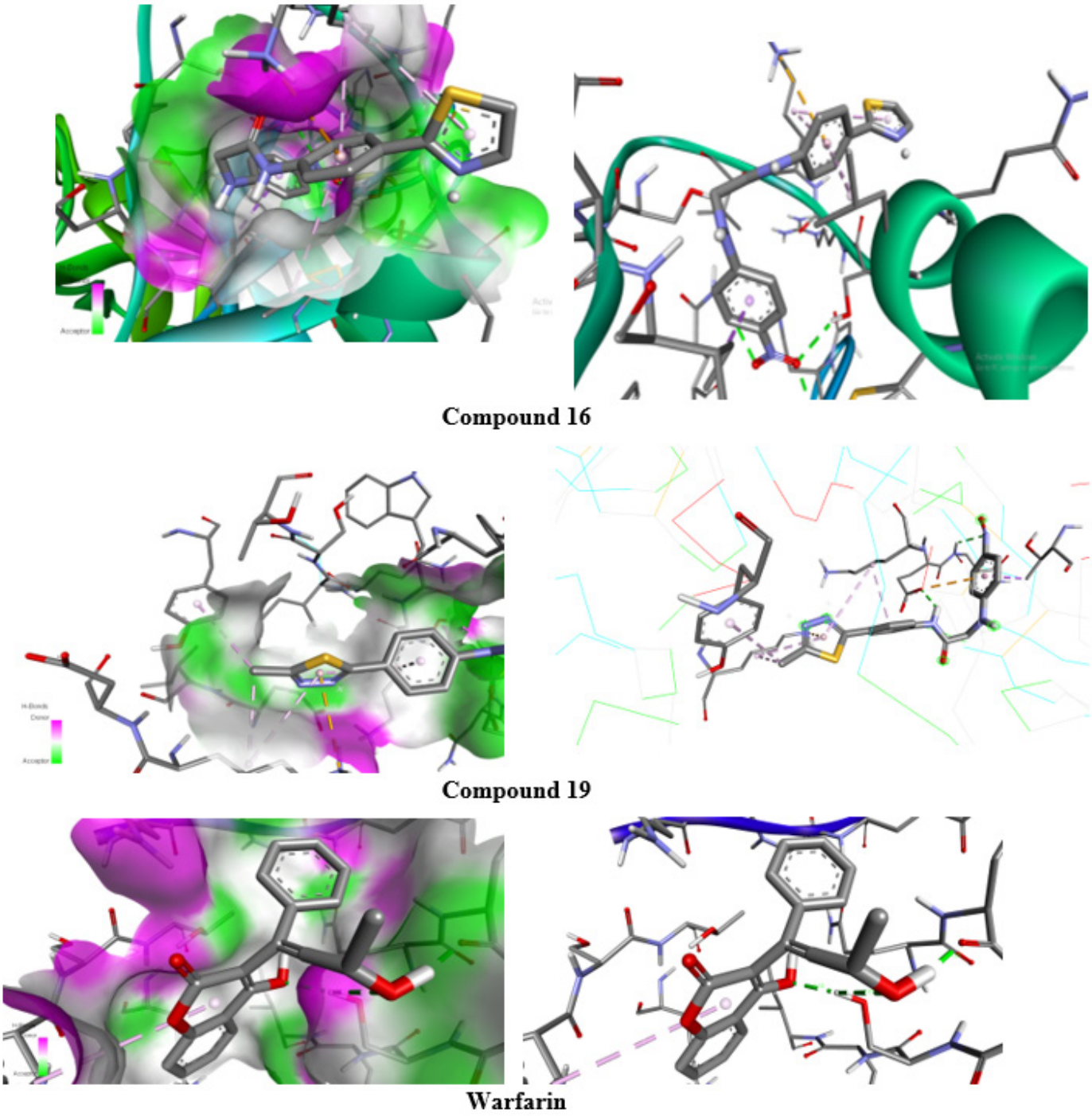


Figure 2: 3D interactions of compounds 4, 7, 15, 16 and 19 with 1DAN Factor VIIa receptor.

The binding affinity of Compound 16 was of -7.8 kcal/mol and exhibited hydrogen bonding with SER 170 H (H), Gln 217 (H), PHE 225 (H) and SER 170B (H), pi sigma interactions with ILE176 (H), pi alkyl interaction with LEU 169 (H), compounds also shown pi cation interaction with LYS 170D (H) residue of receptor. Compound 19 docked with -9.1 kcal/mol of binding energy and had hydrogen interactions with GLU 94 (L), ASN 95 (L), Pi sigma bonding with THR 129 C (H), TYR 71 (T) and pi alkyl interaction with LYS 46 (T) exhibit an alkyl interaction. On the other hand, the standard drug Warfarin demonstrated a binding energy of -7.0 kcal/mol, indicating an alkyl interaction with LYS 15 (T) and a hydrogen bond with LEU 65 (L) and SER

Table 2: **Binding Energies of active compound.**

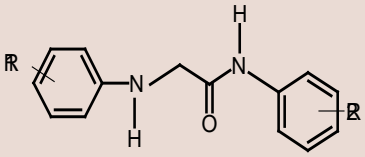
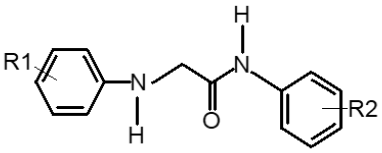
Designed structure	Compound	R1	R2	1 DAN
	4	4-$COCH_3$	4-NO_2	-7.7
	7	3-$COCH_3$	4-NO_2	-7.7
	15	4-$COCH_3$	1,3-thiazole	-8.4
	16	4-NO_2	1,3-thiazole	-7.8
	19	4-NO_2	4-(5-methyl-1,3,4-thiadiazol-2-yl)	-9.1
	Warfarin			-7.0

Table 3: **Inhibitory concentration of active compounds as FVIIa inhibitors.**



Compound	R1	R2	Ki (IM)
			FVIIa
4	4-$COCH_3$	4-NO_2	0.041
7	3-$COCH_3$	4-NO_2	0.048
15	4-$COCH_3$	1,3-thiazole	0.048
16	4-NO_2	1,3-thiazole	0.049
19	4-NO_2	4-(5-methyl-1,3,4-thiadiazol-2-yl)	0.041

67 (L). For the designed acetamide compounds, the electron withdrawing R1 and R2 substitution of benzene ring exhibited good binding affinity and responsible for factor VIIa inhibition. Prothrombin time is typically 14±2. Compounds 4, 7, 15, 16 and 19 among the synthesized compounds also demonstrated 20s, 25s, 16s, 25s and 20s clotting times. These synthetic chemicals were successful in prolonging the typical prothrombin time. The clotting time of a standard warfarin was 60s. Compounds with anticoagulant action were demonstrated by an extension in clotting time.

CONCLUSION

22 novels N-phenyl-2-(phenyl amino) acetamide analogue compounds were synthesized in this research and they were each given a physicochemical and spectroscopic investigation to determine their properties. *In silico* docking research was performed in PyRx to further examine the binding affinities and interactions of anticoagulant drugs to the FVIIa target. *In vitro* anticoagulant activity was performed using prothrombin determination method for these synthesized compounds. *In silico* and *in vitro* studies for these compounds revealed that the compounds 4, 7, 15, 16 and 19 were the most biologically active compound.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

TLC: Thin Layer Chromatography; **FTIR:** Fourier Transforms Infra Red; **NMR:** Nuclear Magnetic Resonance; **CDCl3:** Deuterated Chloroform; **UV:** Ultra-Violet; **TMS:** Trimethylsilane; **2D:** Two Dimensional; **3D:** Three Dimensional; **PDB:** Protein Data Bank.

SUMMARY

The anticoagulant properties of compounds 4, 7, 15, 16 and 19 have been demonstrated both *in vitro* and *in silico*. Good binding affinity to the FVIIa receptor was revealed by *in silico* docking scores and prothrombin time determination and remarkable Ki values support anticoagulation. Schotten Baumann reaction was used as the basis for the synthesis of novel N-phenyl-2-(phenyl-amino) acetamide derivatives.

N-phenyl-2-(phenyl-amino) acetamide derivatives could eventually be employed as therapeutic agents for coagulation disorders.

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